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Quantification of Enterococci and Human Adenoviruses in Environmental Samples by Real-Time PCR

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Pathogenic bacteria and enteric viruses can be introduced into the environment via human waste discharge. Methods for rapid detection and quantification of human viruses and fecal indicator bacteria in water are urgently needed to prevent human exposure to pathogens through drinking and recreational waters. Here we describe the development of two real-time PCR methods to detect and quantify human adenoviruses and enterococci in environmental waters. For real-time quantification of enterococci, a set of primers and a probe targeting the 23S rRNA gene were used. The standard curve generated using *Enterococcus faecalis* genomic DNA was linear over a 7-log-dilution series. Serial dilutions of *E. faecalis* suspensions resulted in a lower limit of detection (LLD) of 5 CFU/reaction. To develop real-time PCR for adenoviruses, degenerate primers and a Taqman probe targeting a 163-bp region of the adenovirus hexon gene were designed to specifically amplify 14 different serotypes of human adenoviruses, including enteric adenovirus serotype 40 and 41. The standard curve generated was linear over a 5-log-dilution series, and the LLD was 100 PFU/reaction using serial dilutions of purified adenoviral particles of serotype 40. Both methods were optimized to be applicable to environmental samples. The real-time PCR methods showed a greater sensitivity in detection of adenoviruses in sewage samples than the viral plaque assay and in detection of enterococci in coastal waters than the bacterial culture method. However, enterococcus real-time PCR overestimated the number of bacteria in chlorinated sewage in comparison with the bacterial culture method. Overall, the ability via real-time PCR to detect enterococci and adenoviruses rapidly and quantitatively in the various environmental samples represents a considerable advancement and a great potential for environmental applications.

Enterococcus spp. are members of the normal floras of the gastrointestinal tract in human and animals and have emerged as a leading cause of nosocomial infection (12, 29). There are two major pathogenic species in human, *Enterococcus faecalis* (*E. faecalis*) and *E. faecium*, with occasional infections being caused by *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. avium*, *E. hirae*, *E. mundtii*, and *E. raffinosus* (11, 32). Enterococcal infections are especially troublesome because of the high level of intrinsic antibiotic resistance (20). The prolonged viability of the naturally occurring *E. faecalis* under simulated deep-sea conditions is a concern with respect to the contamination of the deep ocean with human pathogens and the possibility of sewage-associated microorganisms (3). At recreational beaches, the abundance of enterococci in bathing waters is correlated with the incidence of swimming-related gastroenteritis (4, 5). To protect human health during water recreation, enterococci have been used as an indicator of fecal contamination in California coastal waters since 1999 (36). Thus, rapid detection of enterococci in the environment is of importance in reducing the spread of multiresistant enterococcus and also in assessing the quality of bathing waters. However, conventional methods for enterococcus detection based on cultivation often require 1 to 2 days (12). Additionally, growing bacteria in artificial media contributes to the poor culturability of injured and stressed organisms. Enterococci may also enter a viable

but nonculturable (VBNC) state (26), which may further limit the use of traditional culture methods.

Among human viral pathogens, adenovirus is the only DNA virus in the enteric virus family, hence, the most thermostable virus, and can survive for prolonged periods in environmental waters (13, 16, 17). Adenovirus infections occur year-round, and there is little or no seasonal variation in shedding (1). Adenoviruses have been detected in raw sewage in relatively high frequencies (22, 25), and they were found in environmental sources, including Southern California coastal waters, using molecular methods (23, 33). Pina et al. (33) suggested that adenoviruses be used as an index of human viral pollution because of the concomitancy of adenovirus with other human viruses. There are presently 51 adenovirus serotypes which can be classified into six species, designated species A to F (9). Species F contains two fastidious enteric serotypes, 40 and 41, which are among the leading causes of childhood diarrhea. To prevent human exposure to viral pathogens through drinking water, the Environmental Protection Agency enacted the Information Collection Rule in 1996, which requires all water utilities that serve more than 100,000 households to monitor their source water for viruses. According to the Information Collection Rule, the total culturable viruses had to be detected and enumerated by the total-culturable-virus-assay-most-probable-number (TCVA-MPN) method (14). However, analysis of enteric viruses, particularly adenovirus serotypes 40 and 41, in surface water samples might be greatly underestimated by the TCVA-MPN method due to their fastidious characteristics (6). There have been many efforts attempting to increase the sensitivity of detection for adenovirus, and molecular approaches have emerged as the most promising methods.

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PCR has become an important method for the rapid, sensitive, and specific detection for bacterial and viral agents in the past decade (for examples, see references 7, 30, and 34). More recently, real-time PCR was also applied to quantify the presence of microorganisms in the complex environmental matrices. This method improves the accuracy and sensitivity of traditional PCR by adding a fluorescently labeled probe so that the target gene can be detected and quantified without subsequent verification. Among the various quantitative PCR strategies available, those based on real-time monitoring of the amplification reaction are the most accurate (24). A real-time PCR method targeting 16S rDNA was developed to detect *E. faecalis* in water samples (35). The dynamic range for cell detection spanned 5 logs, and the detection limit was determined to be 6 CFU/reaction without nucleic acid extraction, indicating that real-time PCR is very robust and sensitive in quantification of enterococcus in environmental samples (35). More recently, real-time PCR was applied to detect and quantify *Escherichia coli* (*E. coli*) O157:H7 (21) in food samples and *Enterococcus* spp. in drinking water (15) and to monitor nitrifying bacteria in a municipal wastewater treatment plant (19). Gu et al. (18) also reported real-time PCR quantification of adenovirus in a patient's stool samples. By using control viral samples, sensitivity of detection was demonstrated to be fewer than 10 copies of viral genome per reaction and quantitative linearity was demonstrated to be from 10 to 10⁶ copies of input viral DNA (18).

Human waste contamination of the environment is currently being monitored only with bacterial indicators. However, bacterial and viral contaminations are not necessarily associated and linked with each other (27). Therefore, the fate of various microorganisms through wastewater discharge needs to be further addressed because of the discrepancy between the fates of viruses and bacteria (37). Few methods have been published, particularly with respect to rapid viral quantification, and therefore it is appealing to investigate the presence of human viruses and bacterial indicators using the same approach for common environmental samples, such as sewage, coastal waters, and river waters, to illuminate the relationship between bacterial indicators and human viruses. We selected adenoviruses and enterococci as the target of detection because of their usefulness as an index of coastal pollution. In the present report, we describe the development of real-time PCR methods for quantification of both human adenoviruses and enterococci in environmental samples.

MATERIALS AND METHODS

Bacteria, viruses, cell lines, and culture conditions. *E. faecalis* was purchased from the American Type Culture Collection (ATCC) and was used for optimizing real-time PCR, assessing sensitivity, and generating quantification standards. Cell density of enterococcus in culture and in environmental samples was enumerated using the membrane filtration method (Environmental Protection Agency method 9230C) or enteroalert (IDEX Inc.) and counted as CFU/100 ml or most-probable-number (MPN)/100 ml of sample.

Adenovirus serotype 40 (ad-40), used in real-time PCR optimization and sensitivity assay, was isolated from an environmental sample of Newport Bay, California, by cell culture (HEK-293A, detailed below) in our laboratory and confirmed by sequencing of a 482-bp region in the hexon gene (data not shown). The adenovirus isolate was propagated in HEK-293 cells and purified by a freezing and thawing method. The purified ad-40 particles were titrated using plaque assay on the HEK-293A cells.

The human embryonic kidney cell line (HEK-293A) was obtained from the

University of Southern California (courtesy of Michael Lai) at passage 34 and was used in this study between passage 42 and 50. The cells were grown at 37°C in Dulbecco's modified Eagle's medium with glucose and L-glutamine and supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Human lung carcinoma cell line A549 was obtained from Los Angeles Sanitation District (courtesy of Shawn Thompson) at passage 108 and was used in this study between passage 115 and 123. A549 cells were grown in Ham's F12 medium also containing 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were subcultured at 4- to 5-day intervals with a trypsin-EDTA solution (Cellgro). Both cell lines were used to titrate the viable human adenoviruses in sewage samples by plaque assay.

Adenovirus plaque assay in sewage. Sewage samples (10 ml) were ultracentrifuged at 207,570 × g for 90 min. Pellets were resuspended in 500 µl phosphate-buffered saline (PBS) (pH 7.4) and extracted with 1 volume of chloroform twice to remove cytotoxicants. The supernatants were further clarified by filtering through 0.2-µm-pore-size filters (PES [4 mm, low protein binding]; Whatman). An equal portion of each purified sample was inoculated onto confluent HEK-293A and A549 cells in 6-well plates. The plates were incubated in a CO₂ incubator at 37°C for 1 h and were rocked every 15 min for viral adsorption. Then, inoculated cells were carefully washed once with prewarmed PBS (pH 7.4) and overlaid with warm Dulbecco's modified Eagle's medium, 5% FBS, and 1.25% agarose containing 50 µg/ml gentamicin and 0.25 µg/ml amphotericin B. Cells were incubated at 37°C with 5% CO₂. A second overlay was applied at 6 to 7 days postinfection. Inoculated cell cultures were examined microscopically daily up to 2 weeks postinfection. Plaques were counted as PFU/100 ml sample at 10 days. Highly diluted recombinant adenovirus serotype 5 was used on HEK-293 cells as a positive control, while PBS was used as a negative control. The recombinant adenovirus expresses green firefly protein in infected cells; hereby, the resultant plaque can be confirmed and differentiated under UV microscopy.

Environmental samples. Sewage samples were collected by random sampling in two Southern California sewage treatment plants. Primary effluent was raw domestic sewage after sedimentation. Secondary effluent was treated by activated sludge. Some of the secondary effluents were also treated with chlorine at various locations in the effluent transportation pipeline. The final concentration of chlorine residue was between 2 and 3 ppm in the composite samples.

Coastal water samples were collected from a small estuary, Newport Bay, California, and its tributary, San Diego Creek. The salinity of the water samples was 1 ppt (parts per thousand) in the San Diego Creek site, approximately 15 ppt in mid-Newport Bay, and nearly 30 ppt in lower Newport Bay where the bay water was well mixed with the ocean water. In addition, 31 water samples were collected from various locations and depths from Newport Dunes, a designated recreational beach and recreational vehicle park, in the middle of Newport Bay.

Quantification standards for real-time PCR. To create adenovirus real-time PCR standards, ad-40 hexon gene was amplified by a pair of degenerate primers: Ad1 (5'-TTC CCC ATG GCI AYA ACA C-3') and Ad2 (5'-CCC TGG TAK CCR ATR TTG TA-3') (39). The resultant 482-bp amplicon was cloned into the pCR2.1-TOPO vector (Invitrogen Inc.) and confirmed by sequencing. The purified recombinant plasmid DNA was quantified by UV spectrophotometer and then serially diluted in double-distilled water (ddH₂O) to a final concentration ranging from 2.5 × 10³ to 2.5 × 10⁸ copies of genome equivalent/ml. Four-microliter aliquots of each dilution (10 to 10⁶ genome equivalent/reaction) were used for real-time PCR in triplicates to create the standard curve and used as quantification standards for adenovirus in experimental samples. A new standard curve was run for each real-time PCR.

To generate standards for enterococcus, whole bacterial genomic DNA of *E. faecalis* was extracted using a QIAamp DNA mini kit (QIAGEN Inc.) and quantified by UV spectrophotometer. The concentration of bacterial genomic DNA was converted to a genome equivalent and serially diluted to a range of 2 × 10³ to 2 × 10⁹ copies/ml. A volume of 2.5 µl of each dilution (5 to 5 × 10⁶ copies/reaction) was used in triplicates as a quantitative standard for enterococcus.

Sample preparation for real-time PCR. For real-time PCR of enterococcus, 1 to 10 ml of *E. faecalis* cultures or environmental samples was centrifuged at 6,654 × g for 5 min, and the resultant bacterial pellet was resuspended in 100 µl of lysis buffer (10 mM Tris-HCl [pH 8.3], 100 mM NaCl, 1 mM EDTA, 1% [vol/vol] Tween 20) and 10 µl of 10 mg/ml of proteinase K and incubated at 45°C for 3 h. For coastal water samples, the lysates were extracted with 100 µl phenol-chloroform-isoamyl-alcohol (24:25:1, pH 8.0) and the supernatant was precipitated with ethanol. The resultant DNA pellet was washed with 70% ethanol and dissolved in 30 µl ddH₂O. For sewage samples, the upper liquid phase from phenol-chloroform-isoamyl-alcohol extraction was further purified using a QIAamp DNA Mini kit (QIAGEN) according to the manufacturer's instructions

TABLE 1. Summary of primers and probes for detection of enterococcus and adenovirus

Primer or probe	Sequence	Concn (nM)	T_m (°C)	Assay targets
Enterococcus				
ECF	AGAAATTCCAAACGAACTTG	900	53.1	<i>E. faecalis</i> , <i>faecium</i> , <i>durans</i> , <i>casseliflavus</i> , <i>gallinarum</i> , and <i>hirae</i> ^b
ECR	CAGTGCTCTACCTCCATCATT	300	55.4	
ECP	FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA ^a	250	67.2	
Adenovirus				
AD2	CCCTGGTAKCCRATRTTGTA	300	55.5	Serotypes 1–5, 9, 16, 17, 19, 21, 28, 37, 40, 41, and simian adenovirus 25
AD3	GACTCYTCWGTSAAGYGGCC	300	57.9	
ADP	FAM-AACCAGTCYTTGGTCATGTTRCATTG-TAMRA	200	68.2	

^a FAM, 6-carboxyfluorescein, fluorescence reporter dye; TAMRA, 6-carboxy-tetramethylrhodamine, fluorescence quencher dye.

^b Summarized from a previous publication (15).

to remove PCR inhibitors and interferences. Purified bacterial DNA was eluted with 30 μ l ddH₂O.

For real-time PCR of adenoviruses, 10-ml sewage samples were ultracentrifuged at 207,750 $\times g$ for 90 min. Pellets were resuspended in 500 μ l supernatant and extracted using equal volumes of chloroform twice. Viral nucleic acid was extracted from the concentrates using a QIAamp viral RNA Mini kit (QIAGEN) following the manufacturer's instruction and finally eluted in 30 μ l ddH₂O.

Real-time PCR. Adenovirus primers were designed from the hexon gene encoding the viral structural protein. The oligonucleotide sequences were selected using the Primer Express program (ABI Biosystem Inc.) and manually examined for desired size and melting temperature (T_m) values. The primers and probe sequences were compared to gene sequences in NCBI GenBank using BLAST network service (2). To achieve coverage of a broad range of adenovirus serotypes, the primers and probe were degenerate. Sequences of the oligonucleotides and detectable serotypes are shown in Table 1. Real-time PCR for direct quantification of enterococcus was developed based upon the previous report of Frahm and Obst (15). The enterococcus primers and Taqman probe target the 23S rRNA gene (Table 1).

Real-time PCR was carried out in 25- μ l reaction mixtures consisting of 1 \times TaqMan master mix, primers, TaqMan probe, and template DNA. Concentrations of primers and probe were optimized using a series of concentration matrices to achieve the best amplification efficiency and are presented in Table 1. Samples were amplified and detected using an ABI Prism 7000 sequence detection system (PE Corp.). The PCR amplification conditions were optimized following the manufacturer's recommendations (ABI Biosystems Inc.). The final thermocycling profile for adenoviruses was 95°C for 15, 56°C for 15 s, and 62°C for 30 s with 1 s of automatic increment every cycle for 45 cycles. For enterococcus real-time PCR, the following modified thermocycling profiles were used: 95°C for 15 s and 60°C for 1 min for 40 cycles. All samples were run in triplicate.

RESULTS

Standard curve and sensitivity of real-time PCR. The primers and probes applied in this study were chosen from empirical evaluations of 16 combinations of oligonucleotide sequences targeting the adenovirus hexon gene or enterococcus rRNA gene under various amplification conditions. The amplification conditions tested included variations of oligonucleotide concentration for each primer from 50 to 900 nM, variations of probe concentration from 50 to 250 nM, variations of thermocycling profile according to the primer and probe T_m , and optimization of cycle length and numbers (data not shown). In addition to the goal of achieving optimal real-time PCR settings that give reproducible results with a high sensitivity, the primers and probes were also selected based on their specificity and the range of target detection. Degenerate primers and probes were selected to broaden the range of detectable adenovirus serotypes. The target range of chosen primers

and probes, presented in Table 1, was evaluated using current DNA sequence database (National Center for Biotechnology Information) and literature reviews.

Figure 1 shows the plot of real-time PCR amplification of *E. faecalis* using known copies of genomic equivalent as a standard and a serially diluted bacterial culture as a sensitivity calibrator. The results showed a positive log linear correlation of *E. faecalis* genome copy number and PCR threshold cycle number, stretching a 7-log unit, between 5 and 5 $\times 10^6$ /reaction. The correlation coefficient of the standard curve was constantly 0.99, while the slope was 3.3. The sensitivity of detection for serially diluted known numbers of *E. faecalis* suspension counted by culture was comparable to the standard curve. The lower limit of detection was near 5 CFU/reaction (Fig. 1). Compared to the standard curve, however, a lower slope of the diluted bacteria was noted at the lower concentration range (5 to 50 CFU/reaction). This discrepancy is likely due to the loss of template during DNA preparation from the diluted bacterial samples.

Figure 2 shows the adenovirus real-time PCR plot using known copies of recombinant plasmid DNA inserted with a fragment of ad-40 hexon gene as the standard and a serial dilution of ad-40 viral particles quantified by plaque assay as a

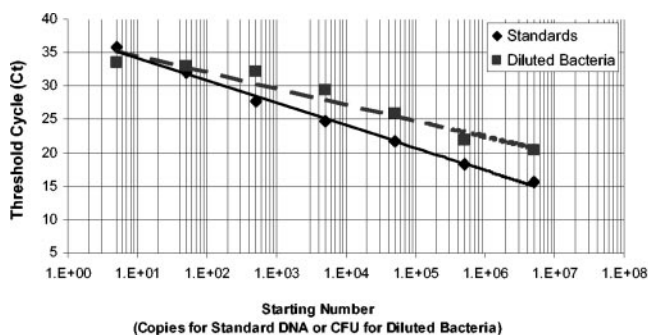


FIG. 1. Standard curve and sensitivity of real-time PCR for detection of enterococcus. Genomic DNA (\blacklozenge) extracted directly from *Enterococcus faecalis* was used as the standard; serially diluted *Enterococcus faecalis* culture (\blacksquare) was used as the sensitivity calibrator. Each data point represents the threshold cycle (Ct) average of samples prepared in triplicate. The solid line represents the standard curve, and the dashed line represents the sensitivity curve.

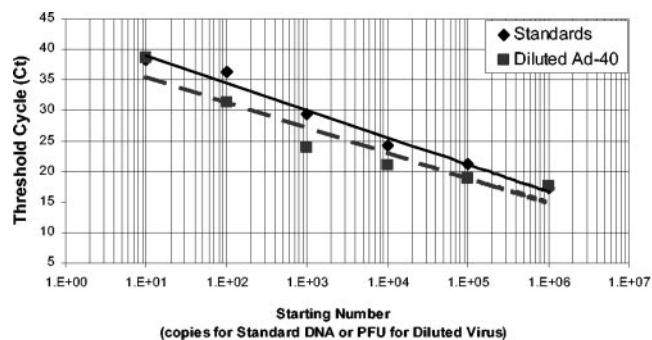


FIG. 2. Standard curve and sensitivity of real-time PCR detection of adenovirus. Plasmid inserted with a fragment of adenovirus hexon gene (♦) was used as the standard; serially diluted adenovirus serotype 40 (■) with known titers was used as the sensitivity calibrator. Each data point represents the cycle threshold (Ct) average of samples prepared in triplicate. The solid line represents the standard curve, and the dashed line represents the sensitivity curve.

sensitivity calibrator. The results showed a positive log linear correlation of hexon gene copy number and PCR threshold cycle number, stretching a 5-log unit, of between 10² and 10⁶ genome equivalent copies/reaction. However, the linearity was poor at the level of 10 genome equivalents/reaction. The correlation coefficient of the standard curve averaged 0.90, and the slope ranged between 3 and 4 for multiple replications of

the standard curve (data not shown). Compared to the real-time PCR for enterococcus, the lower correlation coefficient and variation in the value of the slope in adenovirus real-time PCR may be related to the use of the degenerate primers. The sensitivity of detection for adenovirus was similar to the standard curve using known numbers of infectious viral particles. The lower limit of detection is approximately 100 PFU/reaction (Fig. 2).

Application of real-time PCR for quantification of enterococci in environmental waters. To address the feasibility of real-time PCR in detection of enterococci in environmental waters, we applied this method to various environmental water samples, including sewage, brackish coastal waters, and creek samples, and compared the results with the traditional culture method (Table 2). The first few attempts at direct quantification of enterococci in environmental waters failed to yield satisfactory results due to the sensitivity of real-time PCR assay to inhibitors and interferences in the environmental samples (data not shown). Improvements in bacterial lysis and DNA purification to further clean up real-time PCR templates have been proven successful in the environmental samples (see Materials and Methods). A QIAamp DNA Mini column purification step was necessary to further remove PCR interferences and inhibitors from sewage samples in this study (data not shown).

The results (Table 2) demonstrated that the real-time PCR method was applicable for direct quantification of enterococci in primary, secondary, and chlorinated secondary sewage effluents after sample purification. The bacterial concentration determined by quantification PCR ranged from 10⁵ to 10⁶ genomic copies/100 ml in sewage samples. This is in agreement with the enterococcus counts determined by culture assay in general. However, real-time PCR method significantly overestimated the bacterial concentration in chlorinated secondary effluents, implying that chlorination inactivated the cells but does not completely degrade the target DNA. When real-time PCR was applied to creek and coastal waters, this method also overestimated the bacterial concentrations (Table 2) by 1 to 2 orders of magnitude, especially for samples collected from saline environments such as middle and lower Newport Bay. Of the 31 samples collected from various locations from recreational beaches of Newport Dunes, only 4 samples were detectable by real-time PCR while all samples were below the culture assay detection limit (<10 MPN/100 ml) for enterococcus.

Application of real-time PCR for quantification of adenoviruses in environmental waters. Real-time PCR was applied to quantify adenoviruses in sewage samples in comparison with a plaque assay on A549 and HEK-293A cell lines. Typical viral plaques with diverse morphology were observed on HEK-293A cells but not on A549 cells, indicating that the HEK-293A cell line is more sensitive to infection of adenovirus than A549. Interestingly, number of infectious adenoviruses decreased from 67 or 80 PFU/100 ml in the primary sewage effluents to below detection in the secondary effluents in samples collected from both treatment plants (Table 3). However, there was virtually no difference in viral load between primary effluents and secondary effluents detected by real-time PCR (Table 3). The average number of adenoviral genomes detected in sewage by real-time PCR was 8.1 × 10⁴ viral genomic copies/100

TABLE 2. Comparison of enterococcus quantifications by real-time PCR and culture assay in environmental waters

Sample and date and/or location of collection	Real-time PCR (genomic copies/100 ml)	Culture assay (CFU or MPN/100 ml)
Sewage		
9/15/03		
Primary effluent—plant I	3.5 × 10 ⁵	8.1 × 10 ⁵
Secondary effluent—plant I	1.4 × 10 ⁵	6.0 × 10 ⁵
Secondary effluent—plant II	1.7 × 10 ⁵	3.2 × 10 ⁴
9/30/03		
Primary effluent—plant I	2.6 × 10 ⁶	1.8 × 10 ⁶
Secondary effluent—plant I	3.5 × 10 ⁵	1.4 × 10 ⁵
Primary effluent—plant II	1.6 × 10 ⁵	1.7 × 10 ⁵
Secondary effluent with chlorine—plant II	6.9 × 10 ⁵	0
10/1/03		
Primary effluent—plant I	6.4 × 10 ⁵	2.0 × 10 ⁶
Secondary effluent—plant I	2.1 × 10 ⁵	2.3 × 10 ⁴
Primary effluent—plant II	8.1 × 10 ⁵	4.2 × 10 ⁵
Secondary effluent with chlorine—plant II	5.4 × 10 ⁵	0
Coastal waters		
San Diego Creek	4.5 × 10 ³	8.8 × 10 ²
Middle Newport Bay	2.4 × 10 ³	7.2
Lower Newport Bay	7.3 × 10 ²	59
Newport Dunes (multiple sites; n = 4)	(2.8 ± 2.0) × 10 ²	Below detection ^b
Newport Dunes (multiple sites; n = 27)	Below detection ^a	Below detection

^a Lower limit of detection for real-time PCR is 5 CFU/reaction.
^b Lower limit of detection for enterococci culturing assay using MPN method is 10 MPN/100 ml, while the lower limit of detection using membrane filtration is 1 CFU/100 ml. Only samples from Newport Dunes were assayed by MPN method; the rest of samples were tested using the membrane filtration method.

TABLE 3. Comparison of adenovirus quantification by real-time PCR and plaque assay in sewage samples

Date and location of sewage sample collection	Real-time PCR (viral genomic copies/100 ml)	Plaque assay (PFU/100 ml)
9/30/03		
Primary effluent—plant I	6.6×10^4	67
Secondary effluent—plant I	6.1×10^4	Not detected
Primary effluent—plant II	7.0×10^4	Not detected
Secondary effluent with chlorine—plant II	8.5×10^4	Not detected
10/1/03		
Primary effluent—plant I	7.4×10^4	80
Secondary effluent—plant I	7.4×10^4	Not detected
Secondary effluent with chlorine—plant II	14×10^4	Not detected

ml, which is comparable to the genomic copies of poliovirus in sewage reported by Tsai and Parker (38).

DISCUSSION

We demonstrated real-time PCR methods for enterococcus and adenovirus enabling quantification of both organisms in environmental waters. The higher sensitivity of the real-time PCR method for detection of enterococcus than of adenovirus is likely due to the degenerate primers and probe used for adenovirus real-time PCR, with a larger amplicon and a longer distance between the physical binding locations of the primer and Taqman probe in adenovirus real-time PCR. This tradeoff is made in compensation for the broader target range (i.e., 14 serotypes) and specificity of the PCR.

We chose *E. faecalis* whole genomic DNA instead of plasmid DNA with a single insert of target sequence as a quantification standard, because there are multiple copies of the 23S rRNA gene in each enterococcus genome. Using recombinant plasmid DNA as a standard may compromise the quantification of real-time PCR for enterococcus. The primers and probe for enterococcus real-time PCR used in this study were originally described by Frahm and Obst (15); however, this method was not applied quantitatively to environmental samples in the previous report. A culture enrichment step was required to increase the target bacterial concentration before it was detectable by real-time PCR. The enrichment itself tarnished the characteristic of real-time quantification. In this study, we optimized the ratio of primers to probes from the previously reported 200 nM (forward primer), 300 nM (reverse primer), and 360 nM (probe) to 900 nM, 300 nM, and 250 nM, respectively, to achieve an approximately twofold increase in signal intensity (data not shown). We also improved the extraction and purification method to achieve optimal bacterial DNA purification and removal of PCR inhibitors from water samples. These improvements have allowed this method to be used in environmental samples for quantification of enterococcus in less than 4 h. Compared to the other real-time PCR method reported for *E. faecalis* (35), our current method detects a broader range by targeting the genus rather than an individual species. Therefore, our results are more comparable to those of the culture assay for environmental samples.

The discrepancy between the real-time PCR quantification and enterococcus culture counts was observed in the chlorinated sewage effluents and coastal waters. This is not surprising, because it is well known that chlorination and physical stress may "injure" the cell but may not completely destroy the bacterial genome. In addition, the VBNC state may also account for the discrepancy. VBNC is a survival mechanism of bacteria facing environmental stress conditions (8). Bacteria are no longer able to grow and form colonies on conventional culture media but demonstrate metabolic activity, maintain their pathogenicity, and, in some cases, may return to active growth when optimal conditions are restored (31). Recently, *E. faecalis* has been demonstrated to be able to enter the VBNC state (26). Moreover, a reverse transcription-PCR assay has been established to detect *E. faecalis* pbp5 mRNA to monitor the viability of VBNC (10). VBNC lends further support to criticisms of the traditional methods used to evaluate water quality on the basis of plate counts.

Theoretically, the combination of adenovirus primers and probe covers 14 different serotypes of human adenoviruses. The only nonhuman viral target is simian adenovirus due to the high degree of hexon gene homology between simian adenovirus 25 and other human adenoviruses. Since simian adenoviruses are not expected to be a contamination problem in the urban region of the United States, the application of this method will be unlikely to cause misinterpretation of results of human adenovirus contamination.

In comparison with the plaque assay results obtained using HEK-293A, real-time PCR methods yielded viral counts 3 to 4 orders of magnitude higher. This is similar to a previous study which showed that a much higher number of positive results was obtained by nested PCR than by a cell culture assay (34). The relationship between the copy number of viral genomes and PFU is inconsistent and could be affected by many factors, such as virus strains, the cell line used, sample preparation, and culture conditions. Tsai and Parker (38) obtained 6×10^5 poliovirus for 1 PFU, which is similar to the ratio reported by Metcalf et al. (28). It should be noted that the ratio of viral particles to PFU was not defined for environmental samples in this study. However, the copy number of viral genomes should be proximal to PFU in the calibrators, because purified viral particles from cell culture were used. Therefore, it is important to emphasize that the health implication of detecting viral genome by PCR has multiple complications. It is likely that molecular analysis-based methods tend to overestimate the quantity of infectious human viruses, while the tissue culture-based methods tend to underestimate them. The development of rapid and sensitive genome-based diagnostic tools will improve our ability to illuminate the relationship between viral infectivity and genomic quantity. The method presented here demonstrates that real-time PCR is capable of rapidly quantifying the adenoviral load in environmental waters.

This is the first study that demonstrated the application of degenerate primers and probe for real-time quantification of human adenoviruses in environmental waters. Prospective improvements in DNA extraction and purification are likely to increase the accuracy and sensitivity of these quantification methods and allow a real-world evaluation of efficiency for removing human viral and bacterial pathogens in sewage treat-

ment plants. Real-time PCR will provide a rapid tool to monitor human pathogens in environmental waters.

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